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Drosophila sickle Is a Novel *grim-reaper* Cell Death Activator

John P. Wing,[1,2,6] Janina S. Karres,[1,2,6] Justyne L. Ogdahl,[1] Lei Zhou,[4] Lawrence M. Schwartz,[1,2] and John R. Nambu[1,2,3,5]

[1] Department of Biology
[2] Molecular and Cell Biology Graduate Program
[3] Program for Neuroscience and Behavior
University of Massachusetts at Amherst
Amherst, Massachusetts 01003
[4] Department of Molecular Genetics and Microbiology
Shands Cancer Center
College of Medicine
University of Florida
Gainesville, Florida 32610

Summary

The *Drosophila* genes *reaper*, *head involution defective* (*hid*), and *grim* all reside at 75C on chromosome three and encode related proteins that have crucial functions in programmed cell death (reviewed in [1–5]). In this report, we describe a novel *grim-reaper* gene, termed *sickle*, that resides adjacent to *reaper*. The *sickle* gene, like *reaper* and *grim*, encodes a small protein which contains an RHG motif and a Trp-block. In wild-type embryos, *sickle* expression was detected in cells of the developing central nervous system. Unlike *reaper*, *hid*, and *grim*, the *sickle* gene is not removed by Df(3L)H99, and strong ectopic *sickle* expression was detected in the nervous system of this cell death mutant. *sickle* very effectively induced cell death in cultured *Spodoptera* Sf-9 cells, and this death was antagonized by the caspase inhibitors p35 or DIAP1. Strikingly, unlike the other *grim-reaper* genes, targeted *sickle* expression did not induce cell death in the *Drosophila* eye. However, *sickle* strongly enhanced the eye cell death induced by expression of either an *r/grim* chimera or *reaper*.

Results and Discussion

The Reaper, HID, and Grim proteins can activate caspase-dependent cell death pathways by inhibiting the ability of Inhibitor-of-Apoptosis Proteins (IAPs) to bind caspases and repress their activities [6–8]. Association with IAPs requires the presence of an RHG motif, a 14 amino acid sequence found at the NH_2 terminus of each Grim-Reaper protein. IAP repression also influences cell death activation in mammals, as homodimers of the SMAC/Diablo protein associate with XIAP to relieve caspase-9 inhibition [9–11]. The NH_2 terminus of the mature SMAC/Diablo protein shares sequence similarity to the RHG motif, as both contain a related Ala·Val/Ile·Ala/Pro·Phe/Tyr/Ile tetrapeptide. A similar sequence is also present at the NH_2 terminus of the linker peptide of caspase-9 [12]. The competitive binding to IAPs of caspases and the Grim-Reaper or SMAC/Diablo proteins constitutes a conserved mechanism to regulate cell death activation. Interestingly, Reaper and Grim also contain a second region of similarity, the Trp-block, that is centered around the single Trp residue in these proteins [13]. As Reaper and Grim exhibit both RHG-dependent and RHG-independent cell death activities, the Trp-block may contribute to the full range of their functions.

While the *grim-reaper* genes have central roles in regulating cell death activation, there is evidence suggesting the existence of other crucial death activators in *Drosophila*. Thus, some cell deaths still occur in Df(3L)H99 mutants that lack *reaper*, *hid*, and *grim* [14, 15]. Consequently, we reasoned that other *grim-reaper* genes, perhaps also located at 75C, might mediate these deaths. To test this hypothesis, BLASTP searches were performed on the Predicted Proteins Database (Release 1) using open reading frame sequences in the 75C region. One predicted gene, CG13701, was identified that encodes a small 108 amino acid protein (Figures 1A and 1B) where residues 70–102 are 35% identical to residues 32–65 of Reaper, a region including most of the Trp-block. This gene, which we will refer to as *sickle* (*skl*) (also see Christich et al. and Srinivasula et al., this issue of *Current Biology*), is located at 75C5, 40 kb proximal to *reaper*. Two *sickle* ESTs, RE33794 and RE10476, were identified, and transcription of the *sickle* gene is in the same direction as for *reaper*, *hid*, and *grim.* Further inspection of the Sickle protein sequence revealed that it contains a single Trp-block with 26.7% identity to that of Reaper and 16.7% identity to that of Grim (Figure 1C). Lower levels of similarity are observed between the Sickle Trp-block and HID. Significantly, Sickle also contains an NH_2-terminal RHG motif which is 35.7% identical to those of HID or Grim and 28.6% identical to that of Reaper (Figure 1D). The first half of the Sickle RHG motif, like that of Reaper and Grim, is mostly hydrophobic, while the second half of this RHG motif, like that of HID, is highly hydrophilic. The Sickle RHG motif is unique in containing three consecutive glutamates, including Glu7 and Glu8 that correspond to conserved hydrophobic residues in Reaper, HID, and Grim that participate in association to DIAP1 [16]. These sequence differences likely provide unique functional properties to Sickle, as even the closely related RHG motifs of Reaper and Grim are functionally distinct [13]. The Sickle protein is predicted to have a pKi of 4.67; the first 2/3 of the protein is quite acidic, and the last 1/3 is basic. This contrasts to the basic or neutral pKi predicted for Reaper (pKi = 10.47), HID (pKi = 8.62), and Grim (pKi = 6.9) and further suggests that Sickle may have unique activities. Garnier secondary structure plots [17] predict that residues 62–86 of Sickle will adopt an α-helical conformation (data not shown). This helical region could provide a potential protein interaction interface, perhaps facilitating formation of homodimers or heterodimers with other Grim-Reaper proteins.

[5] Correspondence: jnambu@bio.umass.edu
[6] These authors contributed equally to this work.

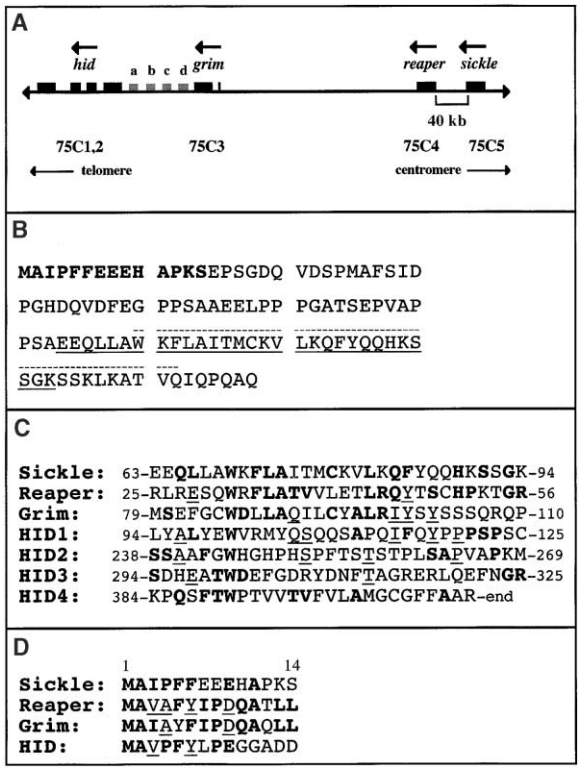

Figure 1. *Drosophila sickle* Is a Novel *grim-reaper* Gene

(A) The *sickle* gene resides 40 kb proximal to *reaper* in a 300 kb region that also contains *hid*, *grim*, and four other genes (gray boxes: a = CG7320, b = CG7313, c = CG5103, d = CG13700). No genes are predicted to reside between *reaper* and *sickle* or between *grim* and *reaper*. The direction of transcription (arrow) for *sickle* is the same as for *hid*, *grim*, and *reaper*, and both the *sickle* and *reaper* genes appear to consist of a single exon (box). Cytology assigned by FlyBase.

(B) The predicted Sickle protein consists of 108 amino acids and includes an NH_2-terminal RHG motif (bold residues) and a Trp-block (underlined residues). The region identified in the BLASTP search with sequence similarity to Reaper is also indicated (¯ above residues).

(C) Alignment of the Trp-blocks of Sickle, Reaper, Grim, and HID indicating their positions within the respective proteins. Positions with identical residues are noted in bold or underlined.

(D) Alignment of the RHG motifs (amino acids 1–14) of Sickle, Reaper, Grim, and HID. Positions with identical residues are noted in bold or underlined.

sickle transcripts were detected beginning at stage 11 of embryogenesis in symmetric dorsal anterior regions in the prospective head region (Figure 2A). Slightly later, *sickle* mRNA was also detected in ventral anterior regions of the head, as well as in cells of the neurogenic ectoderm (Figure 2B). *sickle* continued to be expressed in cells within the developing brain and ventral nerve cord during later stages of embryogenesis (Figures 2C and 2D). Overall, this pattern is similar to that of dying cells within the nervous system, and *sickle* mRNA was also detected within phagocytic macrophages (data not shown) as seen for other *grim-reaper* mRNAs (e.g., [14]). *sickle* gene expression was further analyzed via reverse transcriptase/polymerase chain reaction assays which indicated that *sickle* is expressed in third instar larvae, pupae, and adult heads (see Supplementary Material available with this article online). Expression of *sickle* was also investigated in Df(3L)H99 mutants. Df(3L)H99 is the smallest known deletion that removes *reaper*, *hid*, and *grim*, and the right breakpoint maps close to the *sickle* gene. PCR assays initially indicated that DNA sequences corresponding to the coding region of *sickle* but not *reaper* were still present in homozygous Df(3L)H99 mutants (see Supplementary Materials). Subsequent whole mount in situ hybridizations demonstrated that the *sickle* gene is expressed in Df(3L)H99 mutant embryos (Figures 2E and 2F). Significantly, much more widespread expression of *sickle* mRNA was detected in the nervous system of Df(3L)H99 mutants than in wild-type embryos. This ectopic *sickle* expression is likely the result of an accumulation of *sickle* mRNA in "rescued" neural cells that fail to die in Df(3L)H99 mutants and are not removed via phagocytosis. These data indicate that in the absence of other *grim-reaper* genes, *sickle* is largely insufficient to permit embryonic cell deaths to occur. However, the data do not preclude an essential role for *sickle* in cell death activation. Thus, *sickle* could be crucial for the cell deaths that still take place in Df(3L)H99 mutants, or *sickle* could function predominantly in concert with *reaper*, *hid*, or *grim*. Importantly, as mutations disrupting *hid* or *hid* and *grim* yield much less severe cell death phenotypes than does Df(3L)H99 [18–20], elucidation of *sickle* functions will require determination of the phenotypes of both *sickle*-specific mutants as well as mutants that eliminate *sickle* and other *grim-reaper* genes.

To test *sickle*'s ability to induce cell death, transient transfection assays were performed using cultured *Spodoptera* Sf-9 cells. Survival of the transfected cells was monitored using a LacZ reporter construct. Compared to the empty vector, transfection with the *sickle* expression construct resulted in a dramatic increase in cell death levels, as evidenced by an 18-fold reduction in LacZ expression (Figure 3). Transfection of a *reaper* expression construct also resulted in significant cell loss, although not to the same extent as was observed with *sickle*. The cell death induced by either *sickle* or *reaper* was suppressed ~3- to 6-fold by coexpression of the genes encoding the caspase inhibitory proteins p35 or DIAP1 (Figure 3). These data imply that like other Grim-Reaper proteins, Sickle acts upstream of caspases and induces apoptosis via a mechanism involving inhibition of IAP function (see Christich et al. and Srinivasula et al., this issue of *Current Biology*). The cell death-inducing capabilities of *sickle* were also investigated in *Drosophila*. Surprisingly, P[GMR-*gal4*]-targeted *sickle* expression using P[UAS-*sickle*] strains was ineffective at inducing ectopic cell death in the adult eye. Thus, P[GMR-*gal4*]/P[UAS-*sickle*] animals survived to adulthood, and nearly all exhibited normal eye morphology (Figures 4A and 4B). (A few of these flies did have slightly roughened eyes [data not shown], suggesting weak cell killing effects of *sickle* expression.) This result is in stark contrast to the lethality and complete loss of eye tissue seen for P[GMR-*gal4*]-targeted expression of *reaper*, *hid*, or *grim* [13, 21]. The use of several additional P[*gal4*] strains also failed to yield any evidence for *sickle*-induced ectopic cell death (data not shown). While the

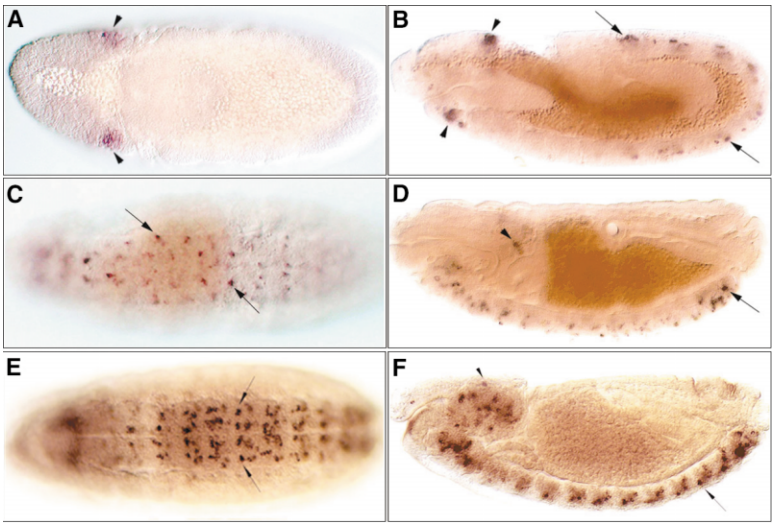

Figure 2. *sickle* Gene Expression in Wild-Type and Df(3L)H99 Mutant Embryos

(A) In situ hybridizations detected expression of *sickle* mRNA in a symmetric cluster of dorsal cells (arrowheads) in the head region of stage 11 embryos. (B) In stage 12 embryos, *sickle* expression was detected in cell clusters within the dorsal/anterior and ventral anterior head region (arrowheads), as well as cells in the neurogenic region (arrows). (C and D) In stage 15 embryos, *sickle* mRNA was observed in cells within the ventral nerve cord (arrows) and brain (arrowhead). (E and F) In stage 15 Df(3L)H99 mutant embryos, ectopic *sickle* gene expression was detected (compare to panels [C] and [D]) in the nerve cord (arrow) and brain (arrowhead). All embryos are presented with anterior to the left; (A) is a dorsal view; (B), (D), and (F) are saggital views; and (C) and (E) are ventral views.

basis for the distinct effects of *sickle* expression in cultured cells and *Drosophila* tissue is not yet clear, cell-specific effects of ectopic *grim-reaper* expression have been previously noted (e.g., [18]).

Because of the synergistic activities of *reaper*, *hid*, and *grim* in embryonic CNS midline [18, 21], we sought to determine if *sickle* might enhance the actions of other *grim-reaper* genes. Since P[GMR-*gal4*]-targeted *sickle* expression failed to induce ectopic cell death, this issue was addressed by coexpression of *sickle* and an *r/grim* chimera or *reaper* in the adult eye. P[GMR-*gal4*]-targeted expression of *r/grim* resulted in viable adults that exhibit a temperature-sensitive loss of eye tissue and pigmentation (Figure 4D; see [13]). In contrast, at either 25°C or 21°C P[GMR-*gal4*]-targeted coexpression of *sickle* and *r/grim* resulted in complete lethality. At 18°C, where the *r/grim* effects are reduced, a few flies coexpressing *sickle* and *r/grim* did emerge, and these exhibited a much greater loss of eye tissue than flies expressing either *sickle* or *r/grim* alone (Figures 4B, 4C, and 4E). Thus, *sickle* exhibited strong synergistic actions with *r/grim*. As expected, the effects of *r/grim*, as well as *r/grim* and *sickle*, were repressed by coexpression of p35; these animals were viable when raised at 25°C and exhibited essentially normal eye size and pigmentation (Figures 4F and 4G). To demonstrate that *sickle*-dependent synergism was not restricted to the *r/grim* chimera, we also examined if P[GMR-*gal4*]-targeted *sickle* expression would enhance cell death induced by P[GMR-*reaper*]. Flies bearing a single copy of P[GMR-*reaper*] exhibited a moderate loss of eye tissue (Figure 4H). In contrast, flies bearing one copy each of P[GMR-*reaper*], P[GMR-*gal4*], and P[UAS-*sickle*], exhibited much more severe eye cell death, with greatly reduced eye size and pigmentation (Figure 4H). This ectopic cell death was repressed by expression of p35 (Figure 4I). Synergistic eye cell death effects were also observed for coexpression of *sickle* and a *g/reaper* chimera, as well as *sickle* and *grim* (data not shown; see Srinivasula et al., this issue of *Current Biology*). These results indicated that *sickle* can potentiate the cell killing effects of *grim-reaper* genes, and they provide the first examples of synergistic action for *grim-reaper* genes outside of the embryonic CNS midline.

In summary, our data demonstrate that *sickle* is a novel member of the *grim-reaper* family of cell death activators and suggest that functional interactions may be a general mechanism underlying the actions of Grim-

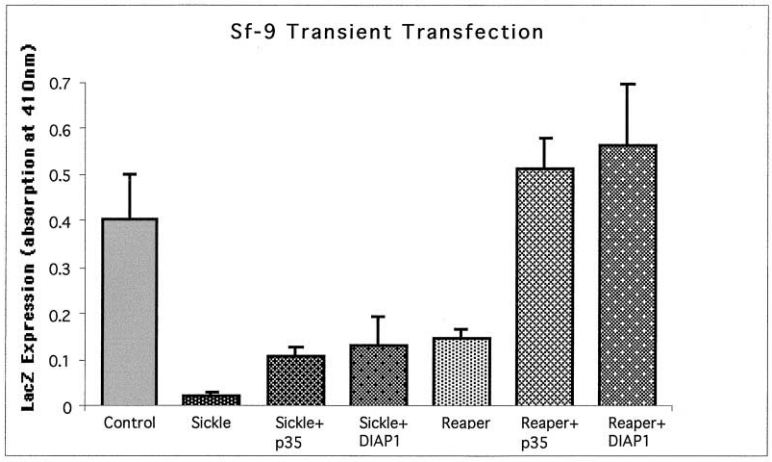

Figure 3. Ectopic *sickle* Expression Efficiently Induced Cell Death in *Spodoptera* Sf-9 Cell Cultures

LacZ expression levels were determined in cultured Sf-9 cells cotransfected with a pIE1-3/*lacZ* reporter construct and (1) pIE1-4 vector, (2) pIE1-4/*sickle*, (3) pIE1-4/*sickle* and pIE1-3/*p35*, (4) pIE1-4/*sickle* and pIE1-4/*diap1*, (5) pIE1-3/*reaper*, (6) pIE1-3/*reaper* and pIE1-3/*p35*, (7) pIE1-3/*reaper* and pIE1-4/*diap1*. Note that, compared to the empty vector, constitutive expression of *sickle* or *reaper* resulted in a strong reduction in LacZ expression corresponding to ectopic cell death. The levels of LacZ expression recovered by 3- to 6-fold upon coexpression of either p35 or DIAP1. The data are derived from seven replicate transfection experiments, and standard errors are indicated.

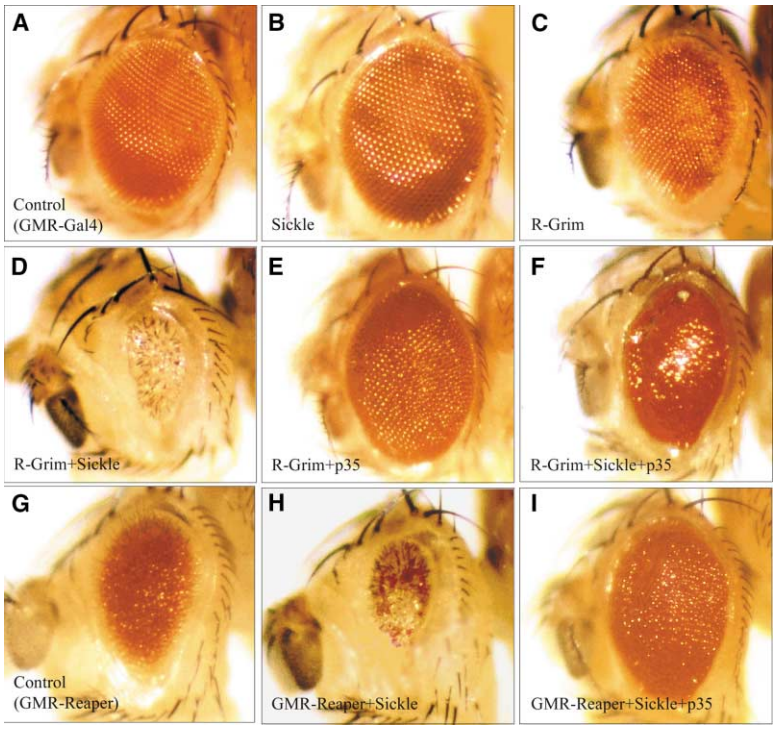

Figure 4. Targeted Expression of *sickle* Alone Did Not Effectively Induce Cell Death in the *Drosophila* Eye, but It Did Potentiate the Effects of *r/grim* and *reaper*

As compared to P[GMR-*gal4*]/CyO (A) flies, the eyes of P[GMR-*gal4*]/P[UAS-*sickle*] flies (B) raised at 25°C did not exhibit eye cell death, as evidenced by the normal eye size and morphology. When raised at 18°C, P[GMR-*gal4*], P[UAS-*r/grim*]/CyO flies exhibited some ectopic eye cell death as indicated by reduced eye pigmentation (C). This cell death phenotype was greatly exacerbated by coexpression of *sickle*, as P[GMR-*gal4*], P[UAS-*r/grim*]/P[UAS-*sickle*] flies (D) raised at 18°C exhibited a strong reduction in eye tissue. The caspase inhibitor p35 repressed *r/grim*-induced cell death, as seen for a P[UAS-*p35*]/+; P[GMR-*gal4*], P[UAS-*r/grim*]/+ fly raised at 25°C (E). p35 coexpression also suppressed the synergistic cell killing effects of *sickle* and *r/grim*, as P[UAS-p35]/+; P[GMR-*gal4*], P[UAS-*r/grim*]/P[UAS-*sickle*] flies (F) were viable and exhibited very slightly roughened eyes that were of approximately normal size. Coexpression of *sickle* and *reaper* also revealed strong synergistic effects, as compared to the relatively mild eye cell death phenotype of P[GMR-*gal4*]/+; P[GMR-*reaper*]/+ flies (G) raised at 25°C, similarly raised P[GMR-*gal4*]/P[UAS-*sickle*]; P[GMR-*reaper*]/+ flies (H) exhibited a much greater loss of eye tissue. This ectopic eye cell death was suppressed by p35, as seen in a P[UAS-p35]/+; P[GMR-*gal4*]/P[UAS-*sickle*]; P[GMR-*reaper*]/+ fly (I).

Reaper proteins. The sequence of the Sickle protein strongly suggests that it has unique RHG motif-dependent and RHG motif-independent functions. Overall, the identification of *sickle* reveals further complexity in the regulation of cell death activation in *Drosophila* and provides additional evidence that these linked genes at 75C be considered a genetic complex [2, 20].

Supplementary Material

All experimental procedures as well as figures for PCR analyses of *sickle* and Sf-9 cell transfections are available at http://images.cellpress.com/supmat/supmatin.htm.

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Accession Numbers

The GenBank accession number for the *sickle* coding region sequence that corresponds to the 327 nucleotide sequence of the *sickle* open reading frame and stop codon generated from cloned *Drosophila* genomic DNA sequenced by us and Flybase is AF460844.